Volume 244, number 1, 26–30 FEB 06766 February 1989

The enzymes of the classical pentose phosphate pathway display differential activities in procyclic and bloodstream forms of *Trypanosoma brucei*

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Received 12 December 1988

The specific activities of each of the enzymes of the classical pentose phosphate pathway have been determined in both cultured procyclic and bloodstream forms of *Trypanosoma brucei*. Both forms contained glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 6-phosphogluconolactonase (EC 3.1.1.31), 6-phosphogluconate dehydrogenase (EC 1.1.1.44), ribose-5-phosphate isomerase (EC 5.3.1.6) and transaldolase (EC 2.2.1.2). However, ribulose-5-phosphate 3′-epimerase (EC 5.1.3.1) and transketolase (EC 2.2.1.1) activities were detectable only in procyclic forms. These results clearly demonstrate that both forms of *T. brucei* can metabolize glucose via the oxidative segment of the classical pentose phosphate pathway in order to produce D-ribose-5-phosphate for the synthesis of nucleic acids and reduced NADP for other synthetic reactions. However, only procyclic forms are capable of using the non-oxidative segment of the classical pentose phosphate pathway to cycle carbon between pentose and hexose phosphates in order to produce D-glyceraldehyde 3-phosphate as a net product of the pathway. Both forms lack the key gluconeogenic enzyme, fructose-bisphosphatase (EC 3.1.3.11). Consequently, neither form should be able to engage in gluconeogenesis nor should procyclic forms be able to return any of the glyceraldehyde 3-phosphate produced in the pentose phosphate pathway to glucose 6-phosphate. This last specific metabolic arrangement and the restriction of all but the terminal steps of glycolysis to the glycosome may be the observations required to explain the presence of distinct cytosolic and glycosomal isoenzymes of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase. These same observations also may provide the basis for explaining the presence of cytosolic hexokinase and phosphoglucose isomerase without the presence of any cytosolic phosphofructokinase activity. The key enzymes of the Entner-Doudoroff pathway, 6-phosphogluconate dehydratase (EC 4.2.1.12) and 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14) were not detected in either procyclic or bloodstream forms of *T. brucei*.

Pentose phosphate pathway; Hexose monophosphate shunt; NADPH; Ribose 5-phosphate; Gluconeogenesis; Entner-Doudoroff pathway; (*Trypanosoma brucei*)

1. INTRODUCTION

Although considerable information about the metabolism of glucose via the glycolytic pathway in trypanosomes is available (review [1]), little is known about other possible metabolic routes for glucose in trypanosomes. An early study of the metabolism of *Trypanosoma rhodesiense* demonstrated the presence of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity in both cultured procyclic and bloodstream forms [2]. However, no evidence was obtained that revealed the fate of the product of the dehydrogenase via either the pentose phosphate or the Entner-Doudoroff pathway [2]. Recent work with procyclic forms of *T. gambiense* and bloodstream forms of both *T. gambiense* and *T. rhodesiense* demonstrated their inability to synthesize purines

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Abbreviations: TIM, triosephosphate isomerase (EC 5.3.1.1); G3PDH, glycerol-3-phosphate dehydrogenase (EC 1.1.1.8); TPP, thiamine pyrophosphate

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

de novo but suggested the operation of an interconverting purine salvage pathway, based on the metabolic fate of isotopically labelled purine bases and nucleosides [3,4]. These results suggest that a source of pentose and deoxypentose phosphates is present in these organisms. Other work also suggests that a functional oxidative pentose phosphate pathway operates in trypanosomes: (i) phosphoribosyltransferase activities for adenine, hypoxanthine and guanine were found to be present in bloodstream forms of *T. brucei* [5] and, (ii) radioactivity from [6-^{14}C]glucose was incorporated into RNA to a 10-fold greater extent than that from [1-^{14}C]glucose [6]. Furthermore, the identification of the enzymes of de novo pyrimidine biosynthesis in various species of trypanosome (review [7]) also supports this hypothesis. Nevertheless, all of the evidence to date for the existence of the pentose phosphate pathway in trypanosomes is indirect.

Here, both procyclic and bloodstream forms of *T. brucei* have been examined directly for the key enzymes of the Entner-Doudoroff pathway and foı the presence of those enzymes that produce ribose 5-phosphate from glucose and constitute the oxidative segment of the classical pentose phosphate pathway. Additionally, we have searched for those enzymes of the non-oxidative branch of the pathway that allow carbon flux from pentose phosphates into the glycolytic pathway and for the gluconeogenic enzyme, fructose-bisphosphatase, that allows net return of glyceraldehyde 3-phosphate from the pentose phosphate pathway to glucose 6-phosphate. The results indicate that both the Entner-Doudoroff pathway and the usual gluconeogenic pathway are absent and that the oxidative segment of the classical pentose phosphate pathway is present. The same results also demonstrate that the enzymes of the non-oxidative segment of the pentose phosphate pathway display differential activities in procyclic and bloodstream forms of *T. brucei*.

2. EXPERIMENTAL

Bloodstream and cultured procyclic forms of *T. brucei* (MITat 1.1, formerly designated 427-12/ICI-060) were used throughout and were grown and purified as described [8–10]. Before use cells were washed three times by suspension and centrifugation with Tris-HCl buffer (20 mM, pH 8.0), containing sucrose (320 mM), Na_2EDTA (1 mM), dithiothreitol (2 mM) and leupeptin (43 μM) and stored on ice.

All assays of enzyme activity except for the lactonase contained 10–20 μl of a stock suspension of freshly isolated cells ($2.8–5.2 \times 10^6$ procyclic or 1×10^7 bloodstream forms/ml of final assay cocktail) as the source of the enzyme to be assayed and were carried out at 30°C in triethanolamine-HCl buffer (50 mM, pH 7.5), containing KCl (50 mM) and Triton X-100 (0.1%) in a final volume of 1 ml. Reactions were monitored spectrophotometrically at 340 nm by following the course of either NADP reduction of NADH oxidation.

It was possible to detect the activity of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) in the presence of NADP and glucose 6-phosphate alone. However, the rate of NADP reduction increased with time, suggesting that the 6-phosphoglucono-δ-lactone formed was being converted to D-ribulose 5-phosphate via the endogenous 6-phosphogluconolactonase and 6-phosphogluconate dehydrogenase activities. Consequently, the activity of glucose-6-phosphate dehydrogenase was obtained by subtracting the measured activity of 6-phosphogluconate dehydrogenase alone from the measured combined activities of 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase. The activity of 6-phosphogluconate dehydrogenase (EC 1.1.1.44) alone was determined in the presence of $MgCl_2$ (5 mM), NADP (0.5 mM) and 6-phosphogluconate (2 mM). The combined activities of 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase were measured in the same way as was 6-phosphogluconate dehydrogenase except that glucose 6-phosphate (2 mM) was also added.

The activity of 6-phosphogluconolactonase (EC 3.1.1.31) was measured in a medium that employed imidazole-HCl as a buffer as in [11] with the sole exception that the assay was conducted at 30°C. Similar results were obtained whether or not the coupling enzymes were desalted. Large blank rates were found to be due to the contamination by lactonase of the commercial preparations of one of the coupling enzymes, glucose-6-phosphate dehydrogenase, when it had been prepared from *Leuconostoc mesenteroides*. The commercial preparations derived from yeast that were tested did not suffer from this problem and were used for all assays of the 6-phosphogluconolactonase reported here. The activity of the lactonase from *T. brucei* was found to be dependent upon the presence of Mg^{2+}.

Transaldolase (EC 2.2.1.2) activity was determined using D-erythrose 4-phosphate (50 μM) and D-fructose 6-phosphate (2 mM) as substrates. The formation of D-glyceraldehyde 3-phosphate was coupled to the oxidation of NADH (100 μM) using TIM (5 U/ml) and G3PDH (1 U/ml). Transketolase (EC 2.2.1.1) activity was measured in the presence of $MgCl_2$ (5 mM) and TPP (25 μM), using D-xylulose 5-phosphate (0.5 mM) as donor substrate and either D-ribose 5-phosphate (1 mM) or D-erythrose 4-phosphate (50 μM) as acceptor. In either case the formation of D-glyceraldehyde 3-phosphate was followed as described above. Ribulose-5-phosphate 3′-epimerase (EC 5.1.3.1) activity was determined using D-ribulose 5-phosphate (0.5 mM) as substrate. The formation of D-xylulose 5-phosphate was coupled to the oxidation of NADH (100 μM) using transketolase (1 U/ml), TIM (5 U/ml) and G3PDH (1 U/ml) in the presence of $MgCl_2$ (5 mM), TPP (25 μM) and D-ribose 5-phosphate (1 mM). A small contamination of commercial transketolase preparations with 3′-epimerase necessitated initiating the reaction with the cell extract in order to correct for the contaminating activity. Ribose-5-phosphate isomerase (EC

5.3.1.6) activity was determined using D-ribose 5-phosphate (1 mM) as substrate. The formation of D-ribulose 5-phosphate was coupled to the oxidation of NADH (100 μM) using ribulose-5-phosphate 3′-epimerase (1 U/ml), transketolase (1 U/ml), TIM (5 U/ml) and G3PDH (1 U/ml) in the presence of $MgCl_2$ and TPP (25 μM).

Fructose-bisphosphatase (EC 3.1.3.11) was measured using D-fructose 1,6-bisphosphate (2 mM) as substrate in the presence of phosphoglucose isomerase (2.5 U/ml), glucose-6-phosphate dehydrogenase (2.5 U/ml), NADP (1 mM) and $MgCl_2$ (10 mM).

The activities of 6-phosphogluconate dehydratase (EC 4.2.1.12) and 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14) were assayed together using 6-phosphogluconate (2 mM) as substrate and coupling the reduction of one of the products of the two enzymes acting in tandem, pyruvate, to the oxidation of NADH (180 μM) using lactate dehydrogenase (2 U/ml) in the presence of $MgCl_2$ (10 mM). This assay circumvents the lack of a commercial source of 2-keto-3-deoxy-6-phosphogluconate but fails to discriminate between the absence of either one or both enzymes in the event of a negative result and measures the activity of only that enzyme present in the lowest amount in the case of a positive result. Nevertheless, the assay is adequate to determine the presence or absence of the pathway as a whole.

When more than one substrate was required for a reaction, the rate in the absence of the last substrate was taken as the blank. In such cases, the reverse order of addition of substrates was used as an additional control for the validity of the assay. Blank rates were observed in a number of the assays that could be traced to small amounts of contaminating compounds that were present in the available commercial preparations of D-erythrose 4-phosphate, D-xylulose 5-phosphate and D-ribose 5-phosphate. These contaminants were eliminated by allowing the blank rate to reach zero or a steady state before adding the final substrate. In every case the blank rate in the absence of the trypanosomal extract also was measured in order to assess the extent, if any, of contamination of the commercial enzymes used in the coupled assays with the enzyme activity actually being measured. The commercial preparations of TIM and G3PDH contributed 2.05 mM $(NH_4)SO_4$ to each assay in which they were used. Solutions of D-xylulose 5-phosphate and D-erythrose 4-phosphate were stored at $-20°C$. All other solutions of substrates were prepared fresh each day.

D-Xylulose 5-phosphate, D-erythrose 4-phosphate, D-ribose 5-phosphate, D-ribulose 5-phosphate, dithiothreitol, leupeptin (synthetic), yeast phosphoglucose isomerase, yeast 6-phosphogluconate dehydrogenase, yeast transketolase and yeast ribulose-5-phosphate 3′-epimerase were purchased from Sigma (Poole, England). NADP, NADH, thiamine pyrophosphate, D-fructose 6-phosphate, D-gluconate 6-phosphate, G3PDH, TIM and glucose-6-phosphate dehydrogenase from yeast and *L. mesenteroides* were obtained from Boehringer (Dublin, Ireland). All enzyme substrates were obtained as their sodium salts. Other chemicals were of the highest available purity.

Protein concentrations were determined by the method of Lowry et al. [12], using bovine serum albumin as a standard. The number of cells in each suspension was determined using a Neubauer improved haemocytometer with a silvered stage; 10^8 procyclic or 10^8 bloodstream forms were found to contain 1.0 and 0.56 mg protein, respectively.

3. RESULTS AND DISCUSSION

Bloodstream and cultured procyclic forms of *T. brucei* were screened for the presence of the enzymes of the pentose phosphate pathway (table 1). Those enzyme activities associated with the oxidative branch of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase, 6-phosphogluconolactonase, 6-phosphogluconate dehydrogenase and ribose-5-phosphate isomerase, were detected in both procyclic and bloodstream forms. Most of these activities were present in greater amounts in bloodstream forms compared to procyclic forms; the enzyme showing the greatest difference (3-fold) between the two forms was the isomerase while the lactonase was present in roughly equal amounts in the two forms. It was not entirely clear why the activity of 6-phosphogluconate dehydrogenase was not detected in an earlier report [2]. One possibility might be that commercial preparations of 6-phosphogluconate at that time consisted of the lactone and we note that these early assays were conducted in the absence of Mg^{2+} and in the presence of EDTA, which would have made the detection of 6-phosphogluconate dehydrogenase difficult if not impossible in extracts of *T. brucei* because of the absolute requirement of the 6-phosphogluconolactonase from this source for Mg^{2+}. Nevertheless, the findings of the present study confirm the predictions of other earlier studies [3–6] and demonstrate clearly that the enzyme activities required to produce D-ribose 5-phosphate for the synthesis of nucleic acids are present in these trypanosomes.

However, two of the enzymes of the non-oxidative branch of the classical pentose phosphate pathway, ribulose-5-phosphate 3′-epimerase and transketolase, were detected only in cultured procyclic forms, while transaldolase activity was found in both forms (table 1). Consideration of the limits of sensitivity of our assays make it clear that ribulose-5-phosphate 3′-epimerase and transketolase activities, if present at all in bloodstream forms, must be reduced by greater than 10- and 100-fold respectively in bloodstream forms compared to their activities in procyclic forms.

These results also indicate that NADPH, CO_2 and D-ribose 5-phosphate are products of glucose metabolism from the oxidative branch of the classical pentose phosphate pathway that are com-

Table 1

Specific activities of the enzymes of the classical pentose phosphate pathway in procyclic and bloodstream forms of *T. brucei*

Enzyme	Specific activity (nmol · min^{-1} · mg cell protein^{-1})	
	Procyclic forms	Bloodstream forms
Glucose-6-phosphate dehydrogenase	12.4 ± 3.8	16.8 ± 5.7
6-Phosphogluconolactonase	40.0 ± 5.7	33.0 ± 8.3
6-Phosphogluconate dehydrogenase	13.2 ± 2.8	21.1 ± 7.3
Ribose-5-phosphate isomerase	54.5 ± 4.3	144 ± 51
Ribulose-5-phosphate 3′-epimerase	5.33 ± 0.84	<0.5
Transaldolase	5.86 ± 0.61	2.61 ± 0.98
Transketolase[a]	50.1 ± 10.8	<0.5
Transketolase[b]	38.6 ± 11.5	<0.5

[a] Ribose 5-phosphate as acceptor substrate
[b] Erythrose 4-phosphate as acceptor substrate

mon to those stages of the life cycle of *T. brucei* that have been examined thus far. However, only procyclic forms are capable of producing significant quantities of hexose phosphate and D-glyceraldehyde 3-phosphate via the classical pentose phosphate pathway because of the differential activities of the enzymes of the non-oxidative segment of the pathway in the two forms of the parasite. Neither form of *T. brucei* was found to be capable of producing D-glyceraldehyde 3-phosphate via the Entner-Doudoroff pathway because of the absence of either one or both of the key enzymes of this pathway, 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase (limit of detection, 0.5 nmol/min per mg protein). The presence of significant quantities of transaldolase activity in the absence of both transketolase and ribulose-5-phosphate 3′-epimerase activities in bloodstream forms of *T. brucei* is puzzling. One possibility is that complete repression of this enzyme is not necessary, simply because it cannot function in the absence of the other two enzymes. Another more likely possibility is that 2-carbon transfer in bloodstream forms occurs in an as yet unknown manner, independent of transketolase and 3′-epimerase activities.

All of the enzyme activities of the pentose phosphate pathway that are present in bloodstream forms have been found to be cytosolic (Nolan and Voorheis, unpublished). It seems likely that these same enzymes as well as the remaining enzymes of the classical pentose phosphate pathway in procyclic forms also will prove to be cytosolic, which would be consistent with the observation that the additional enzyme activities, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase, which are required to metabolize cytosolic glyceraldehyde 3-phosphate to 3-phosphoglycerate, are present in significant quantities in the cytosol as well as in the glycosome [13–17]. The significant absence of fructose-bisphosphatase (limit of detection, 0.5 nmol/min per mg protein) from both forms of *T. brucei* prevents the net conversion of any of the glyceraldehyde 3-phosphate produced back to glucose 6-phosphate by means of this key gluconeogenic enzyme. In addition the existence of the oxidative segment of the classical pentose phosphate pathway in both forms of the parasite provides a rationale for the presence of cytoplasmic hexokinase. Furthermore, phosphoglucose isomerase also would be required in the cytosol for the supply of fructose 6-phosphate to the transketolase and transaldolase reactions in order to cycle pentose phosphates in the procyclic form. However, this explanation would not adequately explain the presence of cytoplasmic phosphoglucose isomerase in bloodstream forms because of our finding that the transketolase is absent in bloodstream forms.

We speculate that differential expression of the respective genes is responsible for the selective absence of the 3′-epimerase and the transketolase from the bloodstream forms. Other possible explanations seem unlikely. For example, it would be very unusual for enzymes that occur in the middle of a pathway, catalyze reactions with equilibrium constants close to unity, occur in large relative amounts when present and have not been shown to allosterically or covalently regulated in any other system should be so regulated in trypanosomes. Furthermore, because these enzymes cannot be detected at all in bloodstream forms, it would be unexpected that organisms as economic as parasites would synthesize the mRNA and then regulate the presence of the enzymes by degrading not some but rather all of the mRNA species before translation could occur. Likewise it seems unlikely that the enzymes themselves would be synthesized and then degraded so quickly that activity would be

completely undetectable with the standard techniques currently available.

The conclusions from this study may be summarized by observing that both procyclic and bloodstream forms of *T. brucei* are incapable of classical gluconeogenesis and also lack the Entner-Doudoroff pathway but do possess the necessary enzyme activities of the oxidative segment of the classical pentose phosphate pathway that are required to produce D-ribose 5-phosphate for nucleic acid synthesis and NADPH for other synthetic reactions. In addition, the presence of a complete complement of the enzymes of the non-oxidative segment of the pentose phosphate pathway in procyclic but not in bloodstream forms of *T. brucei* indicates that significant differences in the cycling of pentose phosphates must occur during the life cycle of trypanosomes.

Acknowledgements: We are grateful to the Wellcome Trust and the Health Research Board of Ireland for financial support and to the Wellcome Trust for the award of a post-doctoral fellowship to C.N.C.

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